Expression of the Parkinson's disease associated gene *alpha-synuclein* is regulated by the neuronal cell fate determinant TRIM32.

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Abstract

Alpha-Synuclein is an abundant neuronal protein which has been associated with physiological processes like synaptic function, neurogenesis and neuronal differentiation but also with pathological neurodegeneration. Indeed, *alpha-synuclein* (*snca*) is one of the major genes implicated in Parkinson's disease (PD). However, little is known about the regulation of *alpha-synuclein* expression. Unveiling the mechanisms that control its regulation is of high importance, as it will enable to further investigate and comprehend the physiological role of alpha-Synuclein as well as its potential contribution in the aetiology of PD. Previously we have shown that the protein TRIM32 regulates fate specification of neural stem cells. Here we investigated the impact of TRIM32 on *snca* expression regulation *in vitro* and *in vivo* in neural stem cells and neurons. We demonstrated that TRIM32 is positively influencing *snca* expression in a neuronal cell line, while the absence of TRIM32 is causing deregulated levels of *snca* transcripts. Finally, we provided evidence that TRIM32 binds to the promoter region of *snca*, revealing a novel mechanism of its transcriptional regulation. On the one hand, the presented data link the PD associated gene *alpha-synuclein* to the neuronal cell fate determinant TRIM32 and thereby support the concept that PD is a neurodevelopmental disorder. On the other hand, they imply that defects in olfactory bulb adult neurogenesis might contribute to early PD associated non-motor symptoms like hyposmia.

Keywords: alpha-synuclein, TRIM32, transcriptional regulation, neuronal differentiation

Introduction

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder, affecting 1-2% of the population over the age of 65 [1-3]. It is characterised by the loss of dopaminergic neurons mainly in the substantia nigra pars compacta, resulting in several motor deficits [4]. The formation of protein aggregates, known as Lewy bodies are considered as a major hallmark of the disease [5]. Braak et al. described in 2003 for the first time six stages that depict the progression of the disease. According to his classification, the first three stages are correlated with a pre-symptomatic phase of PD, while stages 3, 4, 5 and 6 are related to the symptomatic phase, (stage 3 is characterised with both pre-symptomatic and symptomatic phases). More precisely, according to his observations Lewy bodies first appear in the enteric and peripheral nervous system, and in non-dopaminergic structures of the lower brainstem and in the olfactory bulb (OB), and then afterwards they attain the substantia nigra, midbrain, forebrain and neocortex [6].

Although the majority of the PD cases are idiopathic, some known genetic mutations are responsible for the onset of PD in around 10% of all cases [7,8]. 18 genes are now implicated in the pathogenesis of PD, such as the autosomal dominant genes *snca*, *lrrk2* or *vps35*, and the autosomal recessive genes *pink1*, *parkin* or *dj-1* [9]. Patients with point mutations in the *snca* gene present a relatively early age of disease onset, with a mean age of 45.6 years [10]. Alpha-Synuclein (SNCA) is an abundant neuronal protein, mainly linked to synaptic function [5]. Several mutations and multiplications rendered *snca* to be classified as a PD-related autosomal dominant gene responsible for familial cases [9], while also being implicated in a wide range of diseases classified as α-synucleinopathies. SNCA pathology mainly contributes to the intracellular inclusion body formation, the Lewy bodies [4,5]. SNCA containing aggregates appear in the OB of PD patients at an early stage. In addition, the olfactory system is one of the first regions of the brain being affected in PD [11,12] and it is also implicated in hyposmia and anosmia two of the non-motor symptoms of the disease [6]. Therefore, the *snca* gene seems to be an important candidate for the initiation and progression of PD.

Mice overexpressing *snca*, were shown to be greatly affected in certain brain regions, especially the OB and the dentate gyrus (DG). Excess of SNCA was shown to cause a delay in cell cycle entry of neural progenitor cells [13]. Furthermore, its overexpression is associated with increased cell death in the olfactory bulb (OB) and the dentate gyrus (DG) [14-16]. In another study, neurogenesis was investigated *in vivo* and *in vitro* in *snca* transgenic mice (overexpressing *snca*) and in mouse embryonic stem cells (mESC) submitted to differentiation, respectively. For the latter, it was shown that neurogenesis was severely impaired as differentiated mESCs overexpressing *snca* presented a reduced expression of the neuronal marker β-III tubulin. In line with this, the *snca* transgenic mice exhibited a decrease in the number of proliferating cells and neuroblasts in the hippocampus, showing that this gene is linked with the generation of new neurons [14].

The OB comprises one of the two neurogenic regions in the adult forebrain where constantly new neurons are generated. Adult OB neurogenesis is initiated in the subventricular zone (SVZ), from where immature neurons start migrating along the rostral migratory stream (RMS) towards the OB. After reaching the OB immature neuroblasts differentiate into inhibitory interneurons. Eventually, these new neurons are integrated into the existing local neuronal network [17]. This process was shown to be essential for olfaction [18] whereas deregulated adult neurogenesis has an impact on murine social interaction [19] and on olfactory capabilities [20] and finally contributes to Parkinson's disease associated hyposmia [21].

Neurogenesis is regulated by so called cell fate determinants. The protein TRIM32, which belongs to the TRIM-NHL family, functions as such a cell fate determinant. TRIM32 possess an E3 ubiquitin ligase activity. Among the ubiquitination targets are cell cycle regulators like p73 [22] and c-Myc [23]. Additionally, it was also shown that TRIM32 is able to associate with the RNA-induced silencing complex and the RNA helicase DDX6 in order to increase the activity of microRNAs [24]. Previously we have shown that the cell-fate determinant TRIM32 supresses proliferation and is able to induce neuronal differentiation of neural stem cells (NSC) in embryonic [23,25] and adult mouse brain [26]. In the absence of TRIM32, adult neuroblasts of the

subventricular zone-olfactory bulb (SVZ-OB) system undergo extended proliferation and show delayed differentiation and less cell death, leading to an overproduction of adult generated neurons in the OB[26]. Finally, we have recently provided evidence that TRIM32 knock-out (ko) mice, present impaired olfactory capabilities as a result of increased neurogenesis and decreased apoptosis in the OB [20].

Since both TRIM32 and SNCA are involved in adult neurogenesis which is a key characteristic of the olfactory system, we set out to study a putative association between them. Since TRIM32 translocates to the nucleus during neuronal cell fate specification [25,26], we hypothesized that TRIM32 transcriptionally regulates *snca*. So far, only few proteins have been identified to bind directly to the *snca* promoter and regulating its expression [27-29]. SNCA not only has a dominant role in PD pathology, but is also implicated in neurogenesis affecting cell proliferation and death, and the number of newly formed neurons. Obtaining a deeper comprehension of the role of *snca* in the regulation/deregulation of adult neurogenesis, will allow us to come to a closer understanding of the spectrum of the *snca* associated diseases and pave the way towards potential novel therapeutic approaches. In summary, here we describe TRIM32 as a novel regulator for the expression of the PD associated gene *snca*. Furthermore, we present supporting information that TRIM32 is able to bind to the promoter of snca and that the absence of TRIM32 is responsible for abnormal *snca* mRNA levels both *in vitro* and *in vivro*.

Results

TRIM32 regulates transcription of *snca* in a cell type specific manner

Since TRIM32 translocates into the nucleus upon differentiation ([30] and Online Resource 1) we speculated that TRIM32 may regulate the transcription of *snca*. In order to understand the existence of a possible relationship between *snca* and *trim32*, we designed a model based on previously published time series microarray data [31]. Time series measurements can unveil the causal structure of regulatory networks via the identification of associated dynamical models, in contrast to static measurements that usually only allow correlation and statistical (undirected) analysis of gene regulatory networks. Here, dynamical models are defined by differential equations describing the time evolution of certain observed input-output variables. To avoid overfitting, we chose the simplest model class: a first-order linear dynamical system. Hence, given the simplicity of the model, there is a very high confidence that links captured with this tool are in fact true links. The mathematical tools to obtain such systems from data are well described [32]. This methodology was applied to a mouse gene expression time series data set [31] to learn the regulatory network of the *snca-trim32* subsystem (more details are given in the supplementary material). The identified linear models (Fig. 1) reveal a clear regulatory effect of *trim32* on *snca* (85% fit – where 100% is a perfect fit), but not *vice versa* (1% fit). Here, fitness is a measure of agreement between the simulated and real data [32]. In more details, to test whether *trim32* regulates *snca*, we looked for first-order linear dynamical systems that best fitted the data. We obtained $\dot{x}_{snca} = -0.2\, x_{snca} + 0.5\, x_{trim32} + e_1$ with fitness of 85% and where $\dot{x}$ denotes the rate of change of x over time and e represents noise. The high fit indicates a strong evidence that *trim32* regulates snca. Repeating the analysis but with *snca* as the input and *trim32* as the output, the best model had a very poor fitness of 1%.

Based on these *in silico* data, we conducted luciferase assays using a vector encoding for the *snca* intron 1, which is considered to function as main promoter for *snca,* followed by the firefly luciferase gene (Fig. 2a). A basic vector containing solely the firefly luciferase was used as a control. Each construct was then transfected into neuroblastoma (N2a) cells together with

different concentrations of TRIM32. The overexpression of TRIM32 led to a strong increase in the luciferase signal, demonstrating an increase in the transcriptional activity of the *snca* promoter. (Fig. 2b).

When we repeated the same experiment in human embryonic kidney (HEK293T) cells, a low concentration of TRIM32 led to an increased luciferase activity compared to the control. On the other hand, when the concentration was increased, TRIM32 not only lost its ability to induce transcription of *snca*, but it even significantly decreased the transcriptional activity of the *snca* promoter (Online Resource 3). These data indicated that TRIM32 is able to alter the transcriptional activity of *snca* in a concentration and cell type specific manner.

TRIM32 is able to bind to the *snca* promoter sequence

After showing that TRIM32 can activate the *snca* promoter in N2a cells, we next examined if TRIM32 binds to the *snca* promoter. To address this question, we performed chromatin immunoprecipitation (ChIP) assays in N2a cells, using a cocktail of anti-TRIM32 antibodies targeted against either the C- or N-terminal domains of the protein. An IgG antibody was used as negative control and an anti-histone 3 (H3) antibody as positive control. We used several primers covering a ~2.5 kb sequence upstream the transcription start site of the gene (Fig. 3a). Indeed for all sequences investigated we were able to detect a strong interaction of TRIM32 with the *snca* promoter region (Fig. 3b and Online Resource 4).

To further quantify this interaction a set of five pairs of primers was used after ChIP, that cover a 400 bp region upstream of the *snca* transcriptional start site (Fig. 3c). This region was shown to be necessary and sufficient for transcription initiation [33]. With this approach we were able to detect an enrichment of DNA after precipitation of TRIM32, which was strongly above background but somewhat weaker than the interaction of Histone H3 with the *snca* promoter (Fig. 3d, e and Online Resource 5).

To further validate the specificity of the interaction we knocked down TRIM32 in N2a cells by transfecting them with two different shRNA sequences targeting TRIM32. A shRNA scrambled sequence was used as a control. 72 hours after transfection, cells were subjected to ChIP assays and extracted chromatin was analysed via RT-qPCR as described above. Cells transfected with the shRNA scrambled sequence showed a DNA enrichment after precipitation of TRIM32, which follows the same pattern as seen before in the untransfected cells (Online Resource 6a). However, when TRIM32 was knocked down, the enrichment of DNA was dramatically decreased, being almost identical with the negative control (Online Resource 6b). These results indicated that the observed interaction of TRIM32 with the *snca* promoter is specific and indeed the observed interaction can be attributed to the existence of TRIM32. However, these data do not allow us to discriminate between the possibilities that TRIM32 either directly binds to the DNA or whether the interaction is mediated via additional chromatin-binding/associated factors.

snca mRNA expression levels are altered in TRIM32 ko and p73 ko mNSCs

Because *snca* has been described to be upregulated during neuronal differentiation and maturation [34,35], we investigated its mRNA expression levels in mouse NSCs (mNSCs) under stem cell maintenance conditions as well as after induction of differentiation.

To quantify the increase in *snca* expression during differentiation and to investigate whether this is regulated by TRIM32 we used wt and TRIM32 ko mNSCs which were kept under maintenance conditions or subjected to neuronal differentiation for 5 days. In the ko mNSCs indeed the *trim32* transcript was absent as expected (Online Resource 7a). Furthermore, in wt mNSCs *trim32* wass significantly upregulated during neuronal differentiation (Online Resource 7b). The *snca* mRNA expression levels were also upregulated in wt mNSCs subjected to neuronal differentiation (Fig. 4a). However, in TRIM32 ko mNSCs this upregulation of *snca* upon neuronal differentiation was not detectable anymore (Fig. 4a). It is interesting to note that already under maintenance

conditions TRIM32 ko mNSCs had slightly higher levels of *snca* compared to wt, which indicates that compensatory mechanisms, balancing the absence of TRIM32 may be active in these cells.

Since TRIM32 is a direct transcriptional target of TAp73 [22], we hypothesized that the impaired *trim32* expression observed in p73 deficient cells would have an impact on *snca* expression. To address this question we additionally analyzed *trim32* and *snca* mRNA levels in wt and p73 ko mNSCs. The cells were similarly kept under maintenance conditions or were submitted to neuronal differentiation for 1, 3 or 5 days. *trim32* and *snca* levels were strongly upregulated during neuronal differentiation of wt mNSCs (Online Resource 7c and Fig. 4b respectively). However, p73 ko mNSCs presented a blunted induction of *trim32* (Online Resource 7c), which correlated with an almost complete impairment of *snca* induction during mNSC neuronal differentiation (Fig. 4b).

In addition, since we have shown that the TRIM32 dependent regulation of *snca* might be cell type dependent (Fig. 2b and Online Resource 3), we investigated the *snca* mRNA expression levels in wt and TRIM32 ko (Online Resource 8a, b) as well as in wt and p73 ko (Online Resource 6c, d) mouse embryonic fibroblasts (MEFs). We were unable to detect any differences in the *snca* expression levels between the wt and TRIM32 ko MEFs (Online Resource 8b). These observations further strengthen the concept that the regulation of *snca* through TRIM32 is indeed cell type specific. Furthermore, the provided data showing that the absence of only TRIM32 has an effect on the mRNA levels of *snca* during neuronal differentiation, might indicate the strong impact of TRIM32 on *snca* regulation and their importance in balanced neuronal differentiation. wt MEFs express low levels of *snca*, nevertheless, we detected a concomitant small, but significant, decrease in *trim32* and *snca* levels in p73 deficient cells (Online Resource 8c, d). Altogether these data support our hypothesis that TRIM32 regulates the expression of *snca*.

snca is downregulated in the olfactory bulb of TRIM32 deficient mice

The olfactory system is one of the first brain regions impaired in PD. Cells in the OB of PD patients show the typical presence of Lewy bodies which are mainly composed of alpha-Synuclein aggregates [11,12,5]. Moreover, the fact that olfactory bulb (OB) neurogenesis is distorted when TRIM32 is absent [20], prompted us to investigate the mRNA levels of *snca* in different brain regions of adult wt and TRIM32 ko mice (Fig. 5a). As a further control for the specificity of our analysis approach we additionally used a third mouse strain (C57BL/6JOlaHsd) which lacks expression of the *snca* gene due to a small chromosomal deletion [36]. RT-qPCR results confirmed that *trim32* expression is indeed absent in the TRIM32 ko mice and that *snca* is absent in C57BL/6JOlaHsd mice (Fig. 5b, c).

To investigate the potential differential expression of *snca* in the brain of TRIM32 ko mice tissue was dissected from the rostral migratory stream (RMS), the hippocampus (HP), the striatum, the cortex and the olfactory bulb. When the relative *snca* mRNA expression levels were investigated in these brain regions, only in the OB a significant difference was detectable (Fig. 5d). More precisely, the mRNA levels of *snca* were decreased in the OB of TRIM32 ko mice. These results highlight that the impaired neurogenesis observed in the absence of TRIM32 and the involvement of OB in PD might be interconnected through the regulation of *snca* expression via TRIM32.

Discussion

In the present study we provide evidence that the cell fate determinant TRIM32 is able to regulate the transcription of *snca*. Using luciferase assays we obtained indications that overexpression of TRIM32 is positively affecting the transcription of snca. Interestingly, this effect was observed only when the neuronal N2a cell line was used. In non-neuronal HEK293T cells, low concentrations of TRIM32 induced activity of the *snca* promoter while high concentrations inhibited the activity. In addition, in the presented *in silico* data a downregulation of *snca* via *trim32* was predicted though they are based on neuronal dorsal root ganglion cells. Altogether these data indicate that TRIM32 regulates *snca* in a cell type, cell status and concentration dependent manner. This conclusion is further supported by an analysis of *snca* mRNA expression in MEFs and mNSCs.

TRIM32 is associated with several diseases including limb-girdle muscular dystrophy type 2H [37], Bardet-Biedl syndrome [38], cancer [39,40], autism spectrum disorders, attention deficit and hyperactivity disorder and epilepsy, and possibly other neurodevelopmental phenotypes [41-43]. Interestingly, in this context TRIM32 has been described to often have opposing roles in different cell types, e.g. it has been described to be pro- as well as anti-apoptotic [40,39,44-46] and to function as an oncogene as well as a tumour-suppressor [39,40,47]. These opposing functions might be the consequence of cell type and concentration dependent activities of TRIM32 that were described in this study as well.

TRIM32 has been described most intensively to function in neuronal cell fate specification during development and in the adult brain [26,25,23]. In particular, it regulates the generation of new neurons for the olfactory bulb [20]. Interestingly, problems with olfaction (hyposmia) are among the first non-motor symptoms of PD [6] and aggregates of the PD associated protein alpha-Synuclein have been associated with this dysfunction [48,49]. So far there are only few reports revealing proteins binding directly to the *snca* promoter or interacting with DNA response elements in order to form heterodimers and regulate transcription. Nurr1 [29], GATA-1 and GATA-2 [28], ZSCAN21 and ZNF219 [27] have been identified to interact with the promoter region of *snca* and regulate its transcription. Here we provided for the first time evidence that TRIM32 is

interacting with the promoter region of this PD-related gene. Since TRIM32 has no known role of direct DNA binding, it remains to be shown whether it can interact with any of the documented proteins which are bound to the promoter region of the gene, and how this putative interaction might additionally affect transcription. Furthermore, as there is a solid connection between TRIM32 and neuronal defects [41,42], our data further support this relationship. Additionally, a polymorphic microsatellite repeat region (NACP-Rep1), located ~ 10 kb upstream of the transcription start site of the gene, was described to have a regulatory function on *snca* transcription [33,50,51]. The NACP-Rep1 repeat consists of a conserved repetition of nucleotides which acts as a negative modulator of *snca* expression, while the domains flanking the repeat have an inductive role[33]. Whether also this region is regulated by TRIM32 is currently unknown.

We have investigated the transcript levels of *snca* in wt and TRIM32 ko mNSCs under maintenance and neuronal differentiation conditions. wt mNSCs showed an up-regulation of *snca* upon neuronal differentiation, which is consistent with other findings showing the up-regulation of *snca* following differentiation [35,52]. When TRIM32 is missing, a deregulation is occurring in the *snca* mRNA levels of cells submitted to neuronal differentiation. These results were further supported by *in vivo* data, obtained from wt and TRIM32 ko mice where we compared the levels between different brain regions coming from adult tissue. Interestingly, these data, revealed significant differences between wt and ko mice in the mRNA levels of *snca,* only in the OB and not in tissue from any other brain region that was investigated (hippocampus, cortex, striatum and RMS). Already previously *snca* has been implicated with stem cell proliferation and maintenance[53,16,15,14] and our results reinforce the idea that *snca* might indeed represent a crucial player in the balance of neural stem cell maintenance and differentiation during development. p73 has been described as a transcriptional regulator of *trim32* in neural progenitor cells [22]. mNSCs lacking p73 showed a decrease in the mRNA levels of both *trim32* and *snca*. One possible explanation would be that as a consequence of the decreased *trim32* mRNA levels in the p73 ko mNSCs, *snca* levels were also reduced. Interestingly, p73 regulates the expression

of *tyrosine hydroxylase,* an enzyme that is critical for the synthesis of dopamine [54]. Therefore, p73 might be relevant for the development of PD at multiple levels.

In summary, our here presented data suggest that the transcription of the *snca* gene is regulated by TRIM32. Since TRIM32 is highly expressed in the nucleus of cells of the OB, this might represent a regulatory mechanism for the expression of *snca* in the OB. Consequently TRIM32 appears to be an interesting new therapeutic target for modulation of PD. Additionally these data imply that distorted olfactory bulb adult neurogenesis might contribute to early PD associated non-motor symptoms like hyposmia. Finally, by linking the PD associated gene *alpha-synuclein* to the neuronal cell fate determinant TRIM32 the concept that PD is a neurodevelopmental disorder is supported.

Materials and Methods

Reagents and plasmids

For immunolabeling the following antibodies were used: anti-Tuj1 (Covance), anti-NeuN (Millipore) and anti-TRIM32-3149 (Gramsch Laboratories). For ChIP experiments, the following antibodies were used: anti-TRIM32-3150 (Gramsch Laboratories), anti-TRIM32-1137 (Gramsch Laboratories), anti-TRIM32-GS (Genescript) [26,25,55], anti-H3 pan (Diagenode) and IgG (Diagenode).

The following plasmids were used: pEGFP-N1 (Clontech Laboratories), pBABE-FLAG (Daniel Haber), MO93-TRIM32/IRES-EGFP, two commercially available TRIM32 shRNA constructs (TRIM32 shRNA/IRES-GFP into pRNAT-H1.4/Retro vector, Genescript), a commercially available scrambled shRNA construct (shRNA scrambled/IRES-GFP into pRNAT-H1.4/Retro vector, Genescript) and a commercially available *snca* siRNA (Dharmacon). The pGL3-basic luciferase vector, the human intron 1 sequence of α-synuclein subcloned into the pGL3-basic luciferase vector (LUC;Promega, Madison, WI, USA), the pIRES2-EGFP (Clontech) and human wt α-synuclein subcloned into the pIRES2-EGFP were generously provided by Dr. Stefanis, Foundation for Biomedical Research of the Academy of Athens (IIBEAA), Athens, Greece.

Mice

TRIM32 -/- mice were obtained from the Mutant Mouse Regional Resource Centers (MMRRC) (https://www.mmrrc.org/catalog/sds.php?mmrrc_id=11810/011810.html)[26]. C57BL/6JOlaHsd mice were obtained from Harlan laboratories. Animals used in this study for breeding, maintenance and experimental procedures were treated in accordance with the German Federal law on the Care and Use of laboratory animals.

Animal experiments were conducted in agreement with European and Spanish regulations for the protection of experimental animals (Council Directive 86/609/CEE and RD-1201/2005, respectively) with the appropriate institutional committee approval. Mice heterozygous for Trp73 on a mixed background C57BL/6 x 129/svJae [56] were backcrossed to C57BL/6, at least five times, to enrich for C57BL/6 background. Mice were genotyped as described before [56].

Cell culture

Primary NSCs were isolated from single wild type (wt, TRIM32 +/+) and TRIM32 knockout (TRIM32 ko) mouse brains at embryonic day (E) 12.5-14.5. NSCs were cultivated as neurospheres, as described before [24]. Neurospheres were kept under maintenance conditions in DMEM/Ham's F12 medium (PAA) supplemented with 10 ng/mL EGF (Peprotech), 10 ng/mL bFGF-2 (Peprotech), 1 x N2 (Invitrogen), L-Glutamine (PAA), and Penicillin/Streptomycin (PAA). 50% exchange of the maintenance medium with DMEM/Ham's F12 medium (PAA) supplemented with 10 ng/mL bFGF-2 (Peprotech), 1 x N2 (Invitrogen), 1 x B27 (Invitrogen), L-Glutamine (PAA), and Penicillin/Streptomycin (PAA) was used to induce neuronal differentiation. NSCs were kept under differentiation for 5 days and then used for qPCR analysis, or under differentiation for 5 days and then used for immunocytochemical stainings. For immunocytochemical staining, neurospheres were seeded onto coverslips coated with poly-ornithine (Sigma) and Laminin (Sigma).

Neuroblastoma (N2a) cells and Human embryonic kidney (HEK293T, stably expressing the SV40 large T antigen) cells were cultivated on uncoated 10-cm polystyrene tissue culture dishes in DMEM (Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS) (PAA), L-Glutamine (PAA) and Penicillin/Streptomycin (PAA). For transfection followed by luciferase assays, N2a cells and HEK293T cells were seeded onto poly-ornithine-coated 6-well polystyrene tissue culture plates 1 day prior to transfection. For transfection, followed by ChIP assays, N2a cells were seeded on non-coated 10 cm polystyrene tissue culture plates 1 day prior to transfection.

wt and TRIM32 ko mouse embryonic fibroblasts (MEF) were prepared from 12 day old wt and TRIM32 ko embryos as described elsewhere [57]. In total 3 different pairs (from 3 different litters) were generated for the purposes of the experiments.

Independent embryonic cultures of wt and p73 ko mNSCs were initiated by dissecting the OB of at least three different E1.45 embryos, and mechanically dissociating them as previously described [58]. The methods for primary culture of neural precursors cells by the neurosphere assay under mitotic conditions or under neuronal differentiation have been described before [59].

Briefly, differentiation was induced by seeding the mNSCs in differentiation medium (DMEM/Ham's F12, 0.6% glucose 4mM Hepes, 1% Penicllin/Streptomycin, 1% L-Glutamine, 25 µg/ml Insulin, 10% hormone mix and 2% FBS). mNSCs were kept under differentiation for 1, 3 or 5 days and then used for qPCR analysis.

Transfection

N2a and HEK293T cells were transfected using Turbofect (Fermentas) according to manufacturer`s instructions. Each transfection experiment was repeated at least three independent times.

Luciferase assays

Luciferase signal was detected by using the Luciferase Assay System (Promega) according to the manufacturer`s instructions. Briefly, N2a and HEK293T cells were transfected with 4 µg of DNA per well, in 6-well tissue culture plates. Each transfection was performed using either the pBABE-FLAG, the MO93-TRIM32/IRES-EGFP or a combination of the above (pBABE-FLAG and MO93-TRIM32/IRES-EGFP), co-transfected with pGL3-basic luciferase vector or with pGL3-snca intron 1. 72 h post-transfection, cells were lysed in Passive Lysis Buffer (Promega). Luminescence measurements were performed in octuplicate in 96-well plates containing 20 µl lysate per well. For each overexpression sample, the signal obtained from the pGL3-snca intron 1 was normalised to that of the basic vector (pGL3).

Immunocytochemistry

For immunocytochemical staining, cells were fixed with 4% paraformaldehyde in 120 mM PBS, pH 7.4 (4% PFA/PBS) followed by a 15 min permeabilisation using 0.05% Triton X-100 in PBS, at room temperature (RT). Cells were then blocked with 10% FCS in PBS for 1 h at RT and immunofluorescence staining was performed with primary and secondary antibodies diluted in blocking solution. For immunocytochemical staining of the mouse brains, the same protocol was used as described before [26].

Microscopy

Observation of TRIM32 localization on mouse sections and differentiated mNSCs was performed by acquiring images with a Zeiss confocal microscope. Image analysis was performed using ZEN lite (Zeiss), Adobe Photoshop and ImageJ softwares.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed using confluent N2a cells. The High Cell # ChIP kit (Diagenode, Liege, Belgium) was used following the manufacturer's recommendations. Immunoprecipitations were conducted by using the mouse IgG (supplied with the kit), the anti-histone 3 antibody and a cocktail of anti-TRIM32 antibodies (TRIM32-3150, TRIM32-1137 and TRIM32-GS).

PCR

In order to primarily evaluate the eluted DNA which was obtained from the ChIP experiments, we performed PCR, followed by agarose gel electrophoresis. 16 different pairs of primers were used, covering a ~2.5kb area, including exon1, intron 1 and a small region of exon2 of the *snca* gene. The primers which were used for the experiments are listed below, (only representative images of a selection of primers are shown in the Results):

snca-F1:5'-AGCTGCTTGTCCCTTGTTTC-3', snca-R1: 5'-TGGGCACTGGAAGCTAAATC-3', snca-F2:5'-TTTAGCTTCCAGTGCCCAAC-3', snca-R2:5'-GATGCACAGGCTTTGAGACA-3', snca-F3:5'-TGTCTCAAAGCCTGTGCATC-3', snca-R3:5'-GGACCATCACAGGCAGTTTC-3', snca-F4:5'-CGGAAAGGTAAGAGGTGTGC-3',snca-R4:5'-CCATCTGTGGAGCAAGGAAT-3', snca-F5: 5'-ATTCCTTGCTCCACAGATGG-3', snca-R5: 5'-TTTTTACCCCGGAGGAGTCT-3', snca-F6: 5'-CCTCCGGGGTAAAAACAAAT-3', snca-R6: 5'-TTCCCTGCCTCAGTCTGTTC-3', snca-F7: 5'-AACAGACTGAGGCAGGGAAG-3',snca-R7: 5'-CTGTGGGTATCTGGAGCACA-3', snca-F8: 5'-CAGATACCCACAGCCCTCAC-3', snca-R8: 5'-GAACACCTGCTGGTCTCCTG-3', snca-F9: 5'-CAGACGGCAGGAGACCAG, snca-R9: 5'-TTTGCTCCGGCTAGCTAAGA-3', snca-F10:5'-TAGCTAGCCGGAGCAAAGAG-3',snca-R10:5'-TGAAAAAGCAGATGCCACAG-3', snca-F11: 5'-CTGCTTTTTCAAGCGGAAAC-3', snca-R11: 5'-AAAGGCACCCTTCTTCCTCT-3',

snca-F12:5'-GAGGAAGAAGGGTGCCTTTG-3',snca-R12: 5'-GCAGTCTCACTTGAGCACCA-3', snca-F13:5'-TGGTGCTCAAGTGAGACTGC-3',snca-R13:5'-AGAAGGAAGGGAGGGGAACT-3', snca-F14: 5'-CCTCCCTTCCTTCTCCAAAG-3', snca-R14: 5'-GCATCCTCTACCCCATCAAG-3', snca-F15: 5'-TGGGAACTTGATGGGGTAGA-3', snca-R15: 5'-TGGGCACAGTGTTGATTGAT, snca-F16:5'-CAACAATCAATCAACACTGTGC-3', snca-R16:5'-GGCTAAAGATGTATTTTTGCT CCA-3'.

RTq-PCR

Mouse NSCs derived either from wt or TRIM32 ko mice were kept under maintenance conditions or were differentiated for 5 days (5d). Total RNA was extracted by the RNeasy micro kit (Qiagen, Venlo, Netherlands) following the manufacturer's recommendations.

For analysing the *snca* mRNA levels from different brain regions, adult wt, TRIM32 ko and C57BL/6JOlaHsd mice were used. Tissue from the rostral migratory stream (RMS), the cortex, the striatum, the hippocampus and the olfactory bulb (OB) was dissected under a binocular and was snap-frozen. Total RNA was extracted by using the QIAzol lysis reagent (Qiagen, Venlo, Netherlands) according to manufacturer's recommendations.

cDNA was synthesized by using the High Capacity RNA-to-cDNA kit (Applied, Biosystems) according to manufacturer's recommendations. The cDNA synthesis was performed for 1 h at 37^{o}C and the reaction was stopped by heating to 95^{o}C for 5 min.

TRIM32 and *snca* expression levels were evaluated by the Maxima SYBR Green qPCR Master Mix (Thermo Fischer Scientific) following the manufacturer's recommendations. TRIM32 and *snca* levels were evaluated upon normalization to GAPDH levels.

Total RNA from wt and p73 ko MEFs and mNSCs (passage 2) was extracted with TRI reagent (Ambion) and cDNA was prepared using SuperScriptTM II First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. The expression of cell cycle and apoptosis markers was detected by real time quantitative RT-PCR in a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using FastStart Universal SYBR Green Master (ROX)(Roche).

Primers sequences and conditions were described before [22]. The expression levels of mRNA were expressed as $2^{\Delta Ct}$ and normalized to 18S.

The primers which were used for the experiments are listed below:

TRIM32-F: 5'-GCATCCAGGAAGAGCTAG-3'

TRIM32-R: 5'-CTCTACCACTTGACTGTTG-3'

snca-F: 5'-GGTTCCAAAACTAAGGAAGG -3'

snca-R: 5'-CCTCCAACATTTGTCACTTG-3'

GAPDH-F: 5'-CTTTGGCATTGTGGAAGGGC-3'

GAPDH-R: 5'-TGCAGGGATGATGTTCTGGG-3'

Eluted DNA following the ChIP experiments was used for performing qPCR. Enrichment of the DNA was evaluated by the Maxima SYBR Green qPCR Master Mix (Thermo Fischer Scientific) following the manufacturer's recommendations. Results were analysed by using the fold enrichment method. Fold enrichment was calculated by using the formula 2^{-DDCt}, where DDCt is (Ct H3 or TRIM32) – (Ct IgG). Used primers are listed below:

SNCA-F1: 5'- TAGCAGGCAAAGAGCTAGTGG-3'

SNCA-R1: 5'-CAGTCTTTGGAGAAGGAAGGG-3'

SNCA-F2: 5'-CCCTTCCTTCTCCAAAGAACTG-3'

SNCA-R2: 5'-TCCTCTACCCCATCAAGTTCC-3'

SNCA-F3: 5'-GGAACTTGATGGGGTAGAGGA-3'

SNCA-R3: 5'-TGTCACTTAAGGATGGGATGG-3'

SNCA-F4: 5'-CCATCCCATCCTTAAGTGACA-3'

SNCA-R4: 5'-AAAAATCAGTATGCCTTCCCC-3'

SNCA-F5: 5'-ATTTTTCCCCCTCAATTTCCT-3'

SNCA-R5: 5'-AAAGATGTATTTTTGCTCCACAC-3'

Statistics

Statistical analysis was performed by using the Student's t test for single analyses. When

normality tests failed, the Mann-Whitney rank Sum test was used. *P* values of < 0.05 were considered significant.

Regulatory model from microarray data

This section outlines how time series of microarray measurements have been extracted from the dataset of Szpara et al. [31] and used to fit linear models of the form:

$$\dot{x}_{snca} = a_1 x_{snca} + b_1 x_{trim32} + e_1$$

$$\dot{x}_{trim32} = a_2 x_{trim32} + b_2 x_{snca} + e_2$$

where $\dot{x}$ represents the rate of change of x over time, a_i and b_i are parameters to be found, and e represents noise and un-modelled dynamics. First, raw microarray data were downloaded from Szpara et al. [31] and processed using the R functions rma(·) and exprs(·) in the oligo package [60] of http://bioconductor.org/biocLite.R. Expression data, for *snca* and *trim32* in all treatment and control replicates, have been extracted from the complete dataset and mapped back to natural scale from standard $\log_2$-scale. Two single expression time series $data_{gene}$, gene = {*snca*, *trim32*}, were obtained by averaging the expression values at each time point, for t = [2, 5, 12, 24, 40] (in hours). Since the time points were not equally spaced, the time-series have been interpolated using the MATLAB function $\hat{x}_{gene} = pchip(t, data_{gene}, t_{new})$, where t_{new} = [2, 3, …, 39]. The parameters of the models were fitted with prediction error method MATLAB function, based on the algorithms in [32]. In particular, to estimate the transfer function we used the function tfest(Data,Options), where

Options={'InitialCondition'='estimate', 'InitMethod'='n4sid'}

and

Data = iddata(Input,Output)

with Input={trim32,snca} and Output={snca,trim32}.

The fit was computed using compare (System,Output), where System is the identified model.

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Author contributions

MASP: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of the manuscript; NC and JG: model design and interpretation; SFA and MCM: collection and assembly of data, data analysis and interpretation; SN: generation of mNSCs lines; JCS: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of the manuscript.

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Figure legends

Fig. 1 Linear model depicting the dependency of *snca* on *trim32*

Diagram depicting the mRNA expression levels of *snca* (red) and *trim32* (blue) during neuronal differentiation of root ganglion cells, which are plotted in relation to time, where each time point represents the mean and variance of the four replicates of the experimental data taken from Szpara et al. [31]. (a) The left depicts the input to the model (*trim32*). On the right is the output of the model (black), simulated with average initial conditions and driven by *trim32*, and the data from *snca* (red). The fitness of 85% can be verified by the good dynamical agreement of the data (*snca*) and the model driven by *trim32*. The simulation and high fitness empirically justify the simple model class assumption. (b) The reverse direction with *snca* driving *trim32* did not fit a first-order linear system, with the best fit being only 1% fit. The poor fit can be seen on the right side, which compares the simulation of the output of the model driven by *snca* with the data of *trim32*. The simulation also fails to capture the dynamical behaviour of *trim32*

Fig. 2 TRIM32 induces the transcriptional activity of *snca* in N2a cells

(a) Schematic representation of the *snca* gene and the construct used for luciferase assays. The transcription start site is located on exon 2, while the region upstream represents the promoter sequence. White boxes represent untranscribed regions (Exon1 and part of Exon 2), while the black box represents the regions of exon 2 which is transcribed. Intron 1 is depicted as a thick line. The construct being used for the luciferase assays contains the f1 origin of replication and the luciferase gene (luc+). Upstream of luc+, the *snca* intron 1 promoter sequence was inserted. (b) Diagram showing the normalized luciferase activity, in the presence of TRIM32 overexpression. 1.33 µg of TRIM32 were coexpressed together with 1.33 µg of an empty vector and 1.33 µg of the luciferase constructs (basic pGL3 vector or pGL3/snca intron 1) in N2a cells (mean ± SD; n = 6 independent experiments; Mann-Whitney U test, *p ≤ 0.05). 1.75 µg of TRIM32 were coexpressed together with 0.75 µg of an empty vector and 1.75 µg of the luciferase constructs (basic pGL3 vector or pGL3/snca intron 1) in N2a cells (mean ± SD; n = 6 independent

experiments; Mann-Whitney U test, *p ≤ 0.05,). 2.0 μg of TRIM32 were coexpressed together with 2.0 μg of the luciferase constructs (basic pGL3 vector or pGL3/*snca* intron 1) in N2a cells (mean ± SD; n = 3 independent experiments; t-test, *p ≤ 0.05)

Fig. 3 TRIM32 interacts with the promoter region of *snca*

(a) Schematic representation of a fraction of *snca* gene, depicting the five representative out the sixteen primer pairs that were designed for PCR experiments, as an initial evaluation of the ChIP assays. (b) Agarose gel electrophoresis showing PCR results from ChIP eluted DNA, with representative primer pairs of *snca* promoter region. An initial screening of the ChIP experiments was conducted by using PCR, with primers covering an approximate 2.5 kb genomic region. A 100 bp DNA ruler was used on the first lane of both upper and lower images and the samples are indicated. In total sixteen different primer pairs were designed in order to cover the above mentioned large genomic region, but only five representative were chosen to be shown. (c) Schematic representation of a fraction of *snca* gene, depicting the five primer pairs that were designed for RT-qPCR experiments. The five primer pairs are covering the last 400 bp of intron 1, and were used to analyse the DNA retrieved from the immunoprecipitations. (d-e) RT-qPCR results showing the DNA fold enrichment under different immunoprecipitations. RT-qPCR was performed on the eluted DNA derived from chromatin immunoprecipitations either with the IgG (negative control), histone 3 (H3, positive control), or TRIM32 antibodies. Here, two out of five primer pairs are depicting the DNA fold enrichment when H3 or TRIM32 were used for the precipitation, in relation to IgG, meaning the background signal (mean ± SD; n = 3 independent experiments; t-test, *p ≤ 0.05, ** p ≤ 0.001)

Fig. 4 mRNA levels of *snca* are deregulated during differentiation of TRIM32 ko and p73 ko mNSCs

(a) RT-qPCR measuring the relative *snca* mRNA expression levels in wt or TRIM32 ko mNSCs, under maintenance conditions and when cells are submitted for 5 days of neuronal differentiation.

Note how mRNA levels were increased when wt mNSCs were subjected to neuronal differentiation, while TRIM32 ko mNSCs present no such significant increase. Values were normalized to GAPDH levels, (mean ± SD; n = 4 independent experiments with N=4 different cell lines; Mann-Whitney U test, *p ≤ 0.05). (b) RT-qPCR measuring the relative *snca* mRNA expression levels in wt or p73 ko mNSCs, under maintenance conditions and when cells were submitted for 1, 3 or 5 days of neuronal differentiation. Note how mRNA levels were increased in the mNSCs of both genotypes, though the rate of increase was significantly lower in the p73 ko cells. Values were normalized to 18S levels, (mean ± SD; n ≥ 3 independent experiments; t- test, *p ≤ 0.05, **p ≤ 0.001), maint.: maintenance

Fig. 5 mRNA levels of *snca* are decreased in the olfactory bulb of TRIM32 ko mice, but not in any other brain region

(a) Schematic representation of a sagittal mouse brain section, where the different brain regions that were dissected for the RNA isolation are shown. (b) RT-qPCR measuring the relative *trim32* mRNA expression levels in wt, TRIM32 ko and C57BL/6JOlaHsd mice' olfactory bulbs (OB, N_{wt} = 8, $N_{TRIM32\ ko}$ = 11, $N_{C57BL/6JOlaHsd}$ = 6). (c) RT-qPCR measuring the relative *snca* mRNA expression levels in wt, TRIM32 ko and C57BL/6JOlaHsd mice' OB (N_{wt} = 8, $N_{TRIM32\ ko}$ = 11, $N_{C57BL/6JOlaHsd}$ = 6). (d) RT-qPCR measuring the relative *snca* mRNA expression levels in wt, TRIM32 ko and Ola mice' brain regions as indicated. Values were normalized to GAPDH levels, (Mean ± SD; N_{wt} = 8, $N_{TRIM32\ ko}$ = 11 for OB and N_{wt} = 4, $N_{TRIM32\ ko}$ = 4 for the other brain regions; t-test, *p ≤ 0.05, ** p ≤ 0.001), HP: Hippocampus, RMS: Rostral Migratory Stream, OB: Olfactory Bulb